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(54) Title: IMPROVEMENTS RELATING TO DRUG DELIVERY SYSTEMS

(57) Abstract

A two component system designed for use in association with one another comprises (i) a first component that is an antibody fragment capable of binding with a tumour associated antigen, the antibody fragment being bound to an enzyme capable of converting a cytotoxic pro-drug into a cytotoxic drug, (ii) a second component that is a cytotoxic pro-drug convertible under the influence of the enzyme to the cytotoxic drug. This system can be used to control neoplastic cell growth and is designed to improve localisation of the cytotoxic drug. The system utilises benzoic acid nitrogen mustard glutamides convertible to the nitrogen mustard under the influence of carboxypeptidases.

IMPROVEMENTS RELATING TO DRUG DELIVERY SYSTEMS

THIS INVENTION relates to drug delivery systems and is particularly concerned with systems involving interaction between pro-drugs and targetted triggers for the pro-drug.

5   The pharmacological action of drugs used in the treatment of disease is largely determined by differences between cell types in the body and their anatomical location. It is advantageous to have the action of some drugs concentrated in certain tissues. Drugs have been

10   developed which, by virtue of affinities for certain molecular groups have a modified pattern of retention within the body. Attempts to develop drugs which are selectively retained in certain tissues such as cancers have however proved difficult to achieve because of lack

15   of specific molecular configurations in those tissues apart from the various cancer-associated antigens.

One of the main problems in the attack on human cancer is that of selectivity. Most of the drugs which are used are cytotoxic to normal tissues as well as to

20   cancer.

Attempts have been made to improve the treatment of cancer by coupling anti-cancer drugs or radionuclides to antibodies or antibody fragments which have some degree of specificity for cancer associated antigens. The

25   relatively large size of these antibody conjugates results

- 2 -

in their slow diffusion through body spaces and into

tumours. The conjugates are retained to a greater

extent where the antigen is in a higher concentration than

elsewhere. Maximum discrimination in distribution between

5 tumour and non-tumour sites is therefore obtained only

many hours or days after administration. At this time the

concentration of the antibody drug complex has fallen to a

relatively low level both in non-tumour and in tumour

tissue. The effectiveness of therapy depends, in part, on

10 the relative concentration of the active agent in the

tumour and non-tumour tissues and the period of time for

which effective concentration is maintained (often

referred to as 'Areas under the Curve' AUC). The slow

localisation of antibody drug complexes results in time x

15 concentration values which are unfavourable for

therapeutic purposes. Although 2 to 10% of an

antibody dose can be demonstrated to localise in tumour

target in mice the corresponding figure in man is nearer

to 0.1%. There have been attempts in the past to develop

20 anti-cancer drugs which would be in the form of pro-drugs

and which would be activated by enzymes thought to be

present in excess in certain tumours. Unfortunately these

attempts have not succeeded because enzymes have not

proved to be present in tumours either in sufficient

25 quantity or with a sufficiently unique distribution to

confer the necessary specificity of action. Enzymic

activation of pro-drugs to an active form is however well established in principle and some of the widely used anti-cancer drugs, cyclophosphamide and iphosphamide are inactive as administered but converted by liver enzymes

5 into active metabolites.

It has also been shown that an alkylating agent, aniline mustard, is rapidly inactivated by conjugation with a glucuronidase in the liver. The aniline mustard-glucuronide can be converted back into an active form

10 by a glucuronidase. Unfortunately, such an enzyme occurs in sufficient amount only in one type of cancer and that occurs only in experimental mice.

A further example is the release of the alkylating agent phenylene diamine mustard from the

15 peptidyl pro-drug valine-leucine-lysine-phenylene diamine mustard by plasmin. Plasmin is generated by the action of plasminogen activators on plasminogen.

We have recently shown that it is possible to conjugate an antibody or antibody fragment with an enzyme

20 and that the resulting conjugate retains its antibody activity and its enzyme activity. We have now been able to develop this system further so as to provide for the selective delivery and release of a cytotoxic compound at a preselected site in active form.

25 In its broadest aspect, the present invention provides a two-component system designed for use in

- 4 -

association with one another comprising:

(1) A first component that is an antibody fragment capable of binding with a tumour-associated antigen, the antibody fragment being bound to an enzyme capable of
5   converting cytotoxic pro-drug into a cytotoxic drug.

(2) A second component that is a cytotoxic pro-drug convertible to the cytotoxic drug under the influence of the enzyme bound to the antibody fragment of (1) above.

Reference to the word "tumour " in this context
10 and throughout the description is to be understood as referring to all forms of neoplastic cell growth, including leukemias.

In accordance with the present invention, administration of the first component to a mammal bearing
15 a tumour will result, provided that the first component is one that will recognise and bind to the tumour-associated antigen of the tumour, in the selective concentration of the first component in the region of the tumour. After a suitable period of time following the
20 administration of the first component, a proportion of the antibody-enzyme complex will have located and specifically bound to tumour associated antigens.

By selection of an appropriate pro-drug, which will normally exhibit considerably less cytotoxicity than
25 the drug itself, there will be a release of an effective amount of the cytotoxic compound where the antibody-enzyme is at a high concentration, that is, at the target site.

It will therefore be seen that a measure of selective therapy can be secured considerably in excess of the level of selectivity that has been obtainable by

previous methods, particularly those where the cytotoxic compound is directly bonded to an antibody to a tumour-associated antigen or to those methods where reliance is placed upon the existence of endogenous

5 enzymes in sufficient concentration to release the cytotoxic compound from the pro-drug.

The present invention is applicable, in principle, to the delivery of any type of cytotoxic compound in pro-drug form.

10 The characteristics required for each of the components in the system will now be described. The antigenic target or epitope to which the antibody is directed should ideally be a widely expressed component of the cancer cell membrane and one which is not secreted

15 into body fluids. However, experience with immunoscintigraphy indicates that an antigen or epitope may be expressed to some extent by normal cells and that it may be shed by cancer cells or normal cells into body fluids without inhibiting the selective distribution of the antibody to cancer sites provided it is secreted more abundantly than the cancer cells.

20 Considerable work has already been carried out on antibodies and fragments thereof to tumour-associated antigens and antibodies are already readily available that recognise and bind, for example, to human chorionic gonadotrophin (hCG), alfa-feto-protein, carcinoembryonic antigen (CEA), and placental alkaline phosphatase (PLAP), prostate specific antigen, Ca-125 and human milk fat membrane proteins.

- 6 -

The antibody used in the preparation of the antibody-fragment-enzyme conjugate should have a high affinity for the target antigen or epitope but antibodies with a wide range of affinities have been successfully
5   used in immunoscintigraphy. Antibody will generally be of the IgG class but other classes of immunoglobulin are not excluded. They may be polyclonal or more probably monoclonal and should be largely free of impurities. Antibody fragments may be prepared by standard procedures.
10 The fragment of antibody used in the conjugate may possess one or more antigen binding sites and these may be conjugated to enzyme by alternative techniques which include chemical bonding or the production of a hybrid molecule by genetic engineering. Antibody fragments with
15 two antigen binding sites which may have similar or different specificities and may be produced by standard procedures which remove the Fc fragment or they may be constructed by bonding together two fragments having only one antigen binding site each or by genetic engineering.
20 The nature of the chemical bond or bridge between the two fragments should be such that it is not readily broken down in vivo and the bridge between the fragments in a constructed antibody may provide a suitable chemical structure for linkage to the enzyme. Since enzymes are
25 also macromolecules a conjugate comprising intact antibody and enzyme is significantly larger than antibody alone and

this may further delay distribution of the complex to cancer sites. Antibody fragments such as $F(ab')_2$ are smaller and not subject to non-specific binding due to Fc component and are therefore used as the antibody component

5 in the antibody-enzyme conjugate although other antibody fragments, for example Fab_1, are not excluded.

A wide choice of suitable enzymes are available including hydrolases, amidases, sulphatases, lipases, glucuronidases and carboxypeptidases, phosphatases such as carboxypeptidase G2.

10 There are advantages in using a non-mammalian enzyme since if release of the cytotoxic drug from the pro-drug is only securable by the action of a non-mammalian enzyme, premature release of the cytotoxic drug from the pro-drug by endogenous enzymes is avoided.

15 The antibody fragment and the enzyme are normally linked together in a 1:1 ratio as this represents the simplest arrangement. However the present invention· is not restricted to such 1:1 ratios.

In order to link chemically enzyme and antibody

20 satisfactorily in a 1:1 ratio, a heterobifunctional linkage agent which is not labile in physiological conditions has to be employed. Carboxypeptidase G2, for example, and a suitable antibody can be linked together by enriching the antibody with thiol groups and treating the

25 enzyme with a bifunctional agent capable of reacting with these thiol groups, e.g. the N-hydroxy-succinimide ester

- 8 -

of iodoacetic acid NHIA (Rector et al), N-maleimidobenzoyl
succinimide ester MBS (Sigma Ltd.), N-succinimidyl-3-(2-
pyridyl-dithio)-propionate SPDP (Pharmacia Ltd).

However, similar reactions generating a non-labile linkage

5 can be envisaged with other published routes, or
commercially available bifunctional agents: the compound
produced need not be unique in the linkage except insofar
that enzymatic and immunological activities must be
maintained. The linkage methods can be applied to

10 fragmented antibody (e.g. Fab'$_2$-anti-hCG) and to
antibodies or fragmented antibodies directed towards other
known tumour-associated antigens, such as those referred
to above.

15   It will readily be appreciated that the
principle of the present invention can be applied to the
delivery of any cytotoxic drug that can be converted to a
pro-drug for conversion back to the cytotoxic drug under
the selective action of an enzyme. Thus, pro-drugs can be

20 prepared from any of the various classes of anti-tumour
compounds for example:

1.   Alkylating agents (nitrogen mustards), for

example cyclophosphamide, bisulphan, chlorambucil, nitroso

ureas etc.

2.   Intercalating agents, e.g. adriamycin and

5   dactinomycin.

3.   Spindle poisons, e.g. vinca alkaloids.

4.   Anti-metabolites including anti-folates,

anti-purines, anti-pyrimidines or hydroxyurea.

          Our experiments have concentrated at this stage

10   on the use of nitrogen mustards and one pro-drug of

interest for use in the present invention is

bis(2-chloroethyl)-amino benzoic acid where the carboxylic

acid residue is protected by amidation with glutamic acid.

The glutamyl side-chain can then be removed enzymatically,

15   e.g. using carboxypeptidase, to release the nitrogen

mustard.

          Certain of the nitrogen mustards based on

benzoic acid and substituted derivatives thereof,

protected through amidation at the carboxy group e.g. with

20   glutamic acid are new compounds and form a further aspect

of the present invention.

   The new compounds include those of the formula:
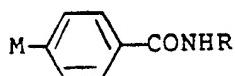
where M is a disubstituted amino "mustard" group and R is

- 10 -

the residue of an α-amino acid RNH_2 and M is a

$$-N\begin{array}{c} CH_2CH_2Cl \\ \\ CH_2CH_2SO_3CH_3 \end{array}$$

$$-N\begin{array}{c} CH_2CH_2SO_3CH_3 \\ \\ CH_2CH_2SO_3CH_3 \end{array}$$

or $\qquad -N\begin{array}{c} CH_2CH_2SO_3CF_3 \\ \\ CH_2CH_2SO_3CF_3 \end{array}$ group.
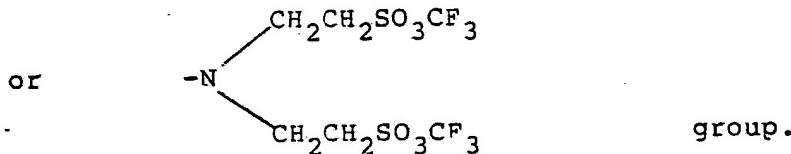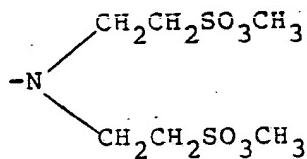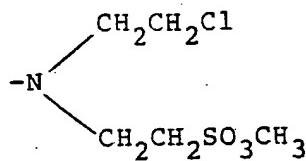
5   The new compounds can be prepared either from
the corresponding compound of the formula:

$$(HOCH_2CH_2)_2N\!-\!\langle\rangle\!-\!CONHR$$
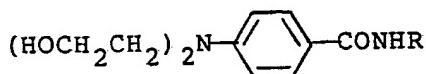
by reaction with a reagent that will replace the HO group
by Cl, CH_3SO_3- or $CF_3.SO_3-$ or by reacting the nitrogen
10   mustard of formula:
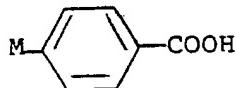
$$M\!-\!\langle\rangle\!-\!COOH$$

or a reactive carboxy derivative thereof with a carboxy protected amino acid RNH_2 and removing the carboxy protecting group.

For the purposes of illustration, these new compounds can be prepared, e.g. where the benzoic acid is to be substituted by a (2-chloroethyl)(2-chloroethyl)amino group from a compound of the formula I which is commercially available, the ethyl protected derivative of p-aminobenzoyl glutamic acid, by the following reactions:

$$H_2N-\underset{}{\bigcirc}-\overset{\overset{O}{\parallel}}{C}-NH-\underset{(CH_2)_2COOC_2H_5}{\underset{|}{C}HCOO-C_2H_5} \quad (I)$$

(i) $CH_2 \overset{O}{\diagdown} CH_2$

$$HO-CH_2CH_2 \diagdown \atop HO-CH_2CH_2 \diagup N-\bigcirc-\overset{\overset{O}{\parallel}}{C}-NH\cdot\cdot\underset{(CH_2)_2COOC_2H_5}{\underset{|}{C}HCOOC_2H_5} \quad (II)$$

(ii) CH_3SO_2Cl

$$ClCH_2CH_2 \diagdown \atop CH_3SO_3CH_2CH_2 \diagup N-\bigcirc-\overset{\overset{O}{\parallel}}{C}-NH-\underset{(CH_2)_2COOC_2H_5}{\underset{|}{C}HCOOC_2H_5} \quad (III)$$

NaOH/HCl

$$ClCH_2CH_2 \diagdown \atop CH_3SO_3CH_2CH_2 \diagup N-\bigcirc-\overset{\overset{O}{\parallel}}{C}-NH-\underset{(CH_2)_2COOH}{\underset{|}{C}HCOOH} \quad (IV)$$
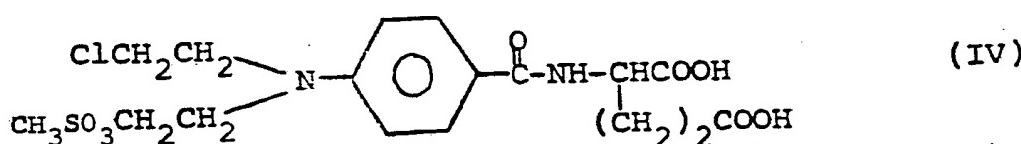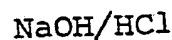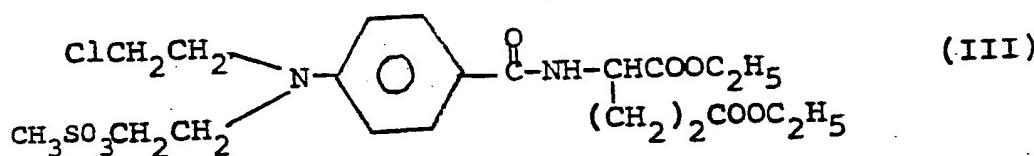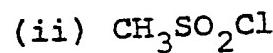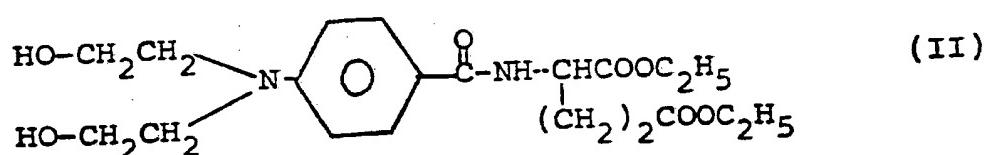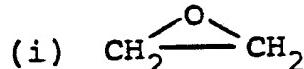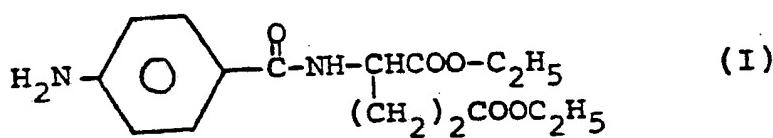
Other novel benzoic acid nitrogen mustard
derivatives are suitable for demonstrating the efficacy of
the invention are:

$$CH_3SO_3CH_2CH_2 \diagdown N \diagup CH_3SO_3CH_2CH_2 \diagup \text{—} \bigodot \text{—} CO\text{—}NH\text{—}CH \diagup CO_2H \diagdown CH_2CH_2CO_2H$$
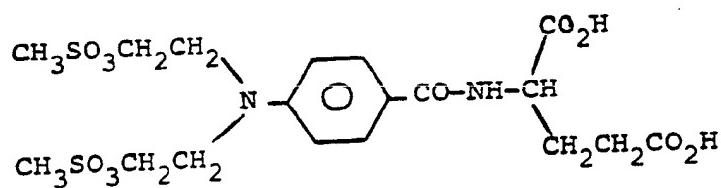
Para N-bis-(mesyl)-amino benzoyl glutamic acid

$$CF_3SO_3CH_2CH_2 \diagdown N \diagup CF_3SO_3CH_2CH_2 \diagup \text{—} \bigodot \text{—} CO\text{—}NH\text{—}CH \diagup CO_2H \diagdown CH_2CH_2CO_2H$$
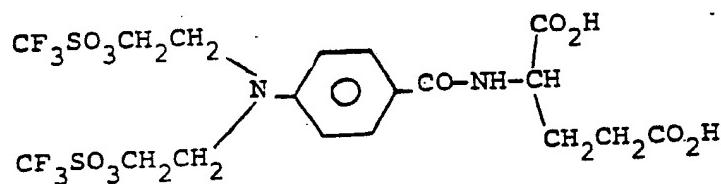
Para N-bis-(triflate)-amino benzoyl glutamic acid

- 14 -

Although the benzoic acid nitrogen mustards are

useful for demonstrating the different aspects of this

system experimentally, the difference in toxicity between

the activated drug and pro-drug in cell culture

5   experiments is only 5 to 10 fold, and this is likely to be

reflected in the in vivo situation. Drugs for clinical

use require this difference to be greater e.g. at least

100 times and preferably, 500-1000 times more toxic than

the pro-drug. Examples of such pro-drugs are

10   anthracyclines where the terminal amino group is

derivatised as an amide with a D-amino acid, and nitrogen

mustards based on p-phenylenediamine with halogen

substituted alkanamido groups. For example, peptidyl

pro-drugs of adriamycin and its analogues, derivatised at

15   the essential amino group (i.e. R'):
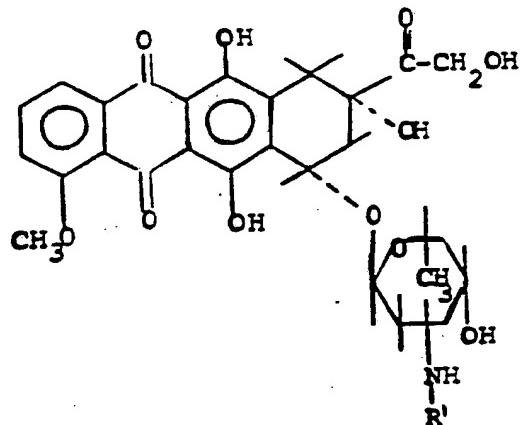
can be used as they can be subsequently released, as

active drugs, by the above-mentioned or similar enzymes,

to open the field to this range of anthracycline drugs.

Use may also be made of other antibody/enzyme conjugates with an appropriate pro-drug. As enzyme, use may be made of endoproteinase

5   Lys-C from lysobacter enzymogenes (available from Boehringer Mannheim) which hydrolyses peptides specifically at the carboxyl group of lysyl residues. This enzyme has a molecular weight of 37,500 and pH optimum 7.7 making it a suitable enzyme to release a

10 nitrogen mustard from a lysyl-rich-peptidyl pro-drug. Clostripain (E C 3.4.22.8) from clostridium histolyticum is a highly specific endoprotease (available from Boehringer Mannheim) which cleaves peptides preferentially at the carboxyl site of L-arginine. Clostripain also

15 cleaves arginine-proline peptide bonds which are not normally attacked by proteases. Its molecular weight of 50,000 and pH optimum, 7.6 make it a suitable candidate for conjugation with an antibody to release nitrogen in partnership with a mustard from an arginyl-prolyl-modified

20 nitrogen mustard.

It is also possible to use an enzyme which releases a toxic nucleoside from its associated nucleotide.

The two-component system of the present

25 invention can be used by sequential administration, the first component, the antibody fragment/enzyme conjugate

being administered first followed by the pro-drug. In

order to secure maximum concentration of the conjugate at

the site of desired treatment, it is normally desirable to

space apart administration of the two components by at

5   least 4 hours. The exact regime will be influenced by

various factors including the nature of the tumour to be

targetted and the nature of the pro-drug, but usually

there will be an adequate concentration of the conjugate

at the site of desired treatment within 24 hours and

10   frequently within 12 or even 8 hours so that the pro-drug

can be administered at this time.

The two components will normally be administered

parenterally and, in accordance with further aspects of

the present invention, there is provided formulations of

15   the conjugate and formulations of the pro-drug, the

formulations being suitable for parenteral administration.

Administration will normally be intravenous and such

formulations are conveniently prepared in isotonic saline

for injection.

20   For the purposes of demonstrating the efficacy

of the present invention, we have worked with a model

system using a monoclonal antibody W14A directed against

human chorionic gonadotrophin (hCG), and $F(ab')_2$ fragments

of the same antibody (2,4) and available from Damon

25   Biotech Ltd., Kirkton Campus, Livingston, EH54, 7BT,

Scotland.

In our experiments, we have used the enzyme
carboxypeptidase G2, a folate degradating enzyme isolated
from Pseudomonas (3), since this is an enzyme that is
known to be capable of removing glutamate residues from

5 folates, methotrexate and from nitrogen mustards derived
from p-amino benzoic acid. The specific conjugates
produced between carboxypeptidase G2 and the $F(ab')_2$
fragments of the monoclonal antibody (W14A) using the
above-mentioned reagents retained enzymatic and

10 immunological activity. The conjugate between
carboxypeptidase G2 and the $F(ab')_2$ fragments of the
monoclonal antibody (W14A) directed against hCG is a novel
compound and forms a further part of this invention.

The pro-drug (Para-N-bis-(2-chloroethyl)-

15 aminobenzoyl glutamic acid) and its activated drug
(benzoic acid mustard) used throughout the Examples was
prepared by the general method described above for the
production of the (2-chloroethyl)-(2-mesylethyl) compound
but replacing the mesyl chloride by thionyl chloride.

20 However, as mentioned above, because of the relatively low
difference in toxicity between the drug and pro-drug, it
is not anticipated that this particular pro-drug will be
used in humans.

- 18 -

Example 1 is a comparison between a conjugate of whole W14A or its F(ab')$_2$ fragment linked to CPG2.

The experiments of Example 1 show the superior properties of the fragment conjugate over the intact W14A

5   conjugate, and the further Examples are concerned only with the use of the fragment conjugate.

EXAMPLE 1

Comparison between conjugates using intact antibody and F(ab')$_2$ conjugates

Intact W14A and F(ab')$_2$ fragment were conjugated

10   with ^{131}I- or ^{125}I-labelled CPG2 using the coupling reagents N-maleimidobenzoyl succinimide ester (MBS), and N-succinimidyl-3-(2-pyridyldithio)-propionate (SPDP), which produce thioether (6) and disulphide bonds (7) respectively. The yields of the coupling reactions, with

15   respect to antibody, were about 27% for SPDP, and 40% for MBS following separation from uncoupled antibody and CPG2 by gel filtration on Ultrogel AcA34 (8).

W14A: ^{131}I-CPG2 conjugates were prepared using CPG2 of specific activity 960 uCi/mg. The specific

20   radioactivity of MBS-linked W14A:CPG2 conjugate was 0.24 uCi/ug and SPDP linked W14A:CPG2 conjugate 0.21 uCi/ug.

- 19 -

$F(ab')_2$: ^{131}-I-CPG2 conjugates were prepared using CPG2 of specific activity 1048 uCi/mg. The specific radioactivity of MBS-linked $F(ab')_2$:CPG2 conjugate was 0.36 uCi/ug, and SPDP-linked $F(ab')_2$:CPG2 conjugate 0.34

5   uCi/ug.

For imaging studies, intact W14A:^{131}-I-CPG2 conjugates were injected i.v. or i.p. into nude rats bearing CC3 choriocarcinoma xenografts (9). $F(ab')_2$:^{131}I-CPG2 conjugates were administered by the i.v.

10   route only. The animals were scanned using a Nuclear Enterprises LFOV gamma-camera.

Quantitative tissue distribution used groups of four nude mice bearing CC3 xenografts. The conjugates were prepared with ^{125}I-labelled CPG2, and injected i.v.,

15   animals receiving approximately 2 uCi/45 ug each. Groups of animals were sacrificed at 24 hour intervals for the collection of tissue samples, which were dissolved in 6M KOH and counted in an LKB model 80,000 "Compugamma" counter.

20   RESULTS

Conjugates labelled only in the CPG2 moiety were used in order that any localising effect determined could be unambiguously attributed to localising of conjugate,

rather than uncoupled antibody, which is difficult to eliminate entirely from conjugate preparations (8). The circulation half-life of native CPG2 is very short, about 3 hours in mice and 1 hour in rats, thus free enzyme would
5   be quickly cleared. The enzyme does not appear to accumulate to a significant degree in any tissue (5).

(i) W14A:CPG2 Conjugates

The gamma-camera images obtained after 24 and 48 hours are presented in Figures 1a and 1b respectively. In
10   the cases of animals injected i.v. with MBS and SPDP linked conjugates the tumour site was clearly defined, confirming that localisation had occurred, but there was also substantial hepatic uptake. As expected, native enzyme was cleared rapidly from the circulation and no
15   tumour or hepatic uptake was observed.

The SPDP-linked conjugates were cleared from the tumour more rapidly than MBS-linked material, suggesting that the disulphide linkage was less stable. Previous reports have suggested that disulphide bonds are labile
20   in vivo (10, 11) and this method of linkage would appear to be unsuited to this application.

Animals injected i.p. with conjugate showed no tumour uptake, with SPDP-linked conjugate cleared by 48

hours and MBS-linked conjugate retained in the liver and
blood pool. Pharmacokinetic studies (unpublished data)
suggest that i.p. injection results in a slow release of
conjugate into the circulation with peak levels only 30%
5 of a comparable i.v. dose.

(ii) $F(ab')_2$:CPG2 Conjugates

The gamma-camera images obtained 24 and 72 hours
after i.v. injection are presented in Figures 2a and 2b.
MBS-linked conjugate showed a sharp tumour image with
10 little or no uptake in the liver. SPDP-linked conjugate
showed some uptake by the liver at 24 hours, but material
was cleared by 72 hours. There was markedly less tumour
uptake and these results reaffirmed the unsuitability of
SPDP-linked conjugates for tumour imaging.

15 (iii) Quantitative Tissue Distribution of Antibody:Enzyme
Conjugate

The tissue distribution of MBS-linked W14A and
$F(ab')_2$:CPG2 conjugates in tumour, blood and liver are
presented in Table 1.

- 22 -

TABLE 1 - Major Tissue Uptake of W14A, and its $F(ab')_2$
Fragment compared with W14A:CPG2 and $F(ab')_2$:
CPG2 Conjugates

	24 hr	48 hr	72 hr
Tumour			
W14A	1.48	0.94	0.85
W14A:CPG2	0.23	0.21	0.22
$F(ab')_2$	0.76	0.39	0.29
$F(ab')_2$:CPG2	1.10	0.83	0.70
Blood			
W14A	2.01	1.09	0.63
W14A:CPG2	0.87	0.41	0.34
$F(ab')_2$	0.83	0.27	0.10
$F(ab')_2$:CPG2	1.52	0.58	0.27
Liver			
W14A	0.98	0.59	0.33
W14A:CPG2	0.27	0.11	0.09
$F(ab')_2$	0.33	0.13	0.06
$F(ab')_2$:CPG2	0.34	0.14	0.09

Values are calculated as percentage of the
injected dose per gram of tissue and compared with intact
W14A and $F(ab')_2$ fragment controls. The results can be
summarised:-

The levels of $F(ab')_2$:CPG2 in BLOOD were

- comparable to those found for W14A:CPG2 conjugate

- about 50% of the level of intact W14A

- about 3-fold higher than those of native $F(ab')_2$

5   fragments.

The TUMOUR uptake of $F(ab')_2$:CPG2 was

- comparable with that obtained with native W14A

- 3-fold higher than the levels attained with

   W14A:CPG2 conjugate or free $F(ab')_2$.

10 The LIVER uptake of $F(ab')_2$:CPG2 was lower than that of

     native W14A

- similar to levels attained with intact

   W14A:CPG2 conjugate or $F(ab')_2$ fragments.

The level of uptake of $F(ab')_2$ conjugates in lung, spleen,

15 kidney, colon and muscle followed a similar pattern

to that of liver, with levels being similar to native

$F(ab')_2$ fragments or W14A:CPG2 conjugate.

    This demonstrates the advantages obtained by

using $F(ab')_2$:CPG2 conjugates rather than the W14A:CPG2

20 conjugate, as the ratio of the fragment conjugate in the

tumour compared to the blood is 3 fold higher than for the

W14A conjugate, though the background level of the

fragment conjugate in other organs is similar to

W14A:CPG2.

- 24 -

EXAMPLE 2

In vitro cytotoxicity of Pro-drug + Active Drug

This shows the toxicity relationship between a cytotoxic compound and its less toxic pro-drug.

5   The 50% growth inhibiting dose (ID50) for MAWI cells (colorectal cancer) in vitro (5×10^4 cells/ml was 25 um with the active drug (benzoic acid mustard). The maximum inhibition that could be obtained with the pro-drug was 7% at a concentration of 400 um.

10   The ID50 for the pro-drug on MAWI cells with carboxypeptidase G2 (CPG2) present in the medium at a concentration of 6 units was 30 uM (i.e. very similar to that for the active drug).

For LS174 (colorectal cancer) cells the ID50 for active drug was 30 uM and also for pro-drug in the

15 active drug was 30 uM and also for pro-drug in the presence of CPG2 at 6 units ml. The pro-drug alone at 400 uM inhibited growth by 15% compared with untreated control cultures.

EXAMPLE 3

20 Distribution of Pro-drug and Active Drug in vivo, in plasma at 24 and 48 hours post antibody-enzyme conjugate

Nude mice bearing human choriocarcinoma (CC3) tumour received 29 units of CPG2 conjugated to anti-HCG (W14 Fab$_2$) and after 24 or 48 hours received pro-drug (41

25 uM/kg). Control mice received the same dose of pro-drug but no CPG2-W14 (Fab$_2$) conjugate.

- 25 -

In the control mice the plasma concentration of pro-drug fell from 5 uM at 5 mins post injection to 0.8 uM at 3 hours; the active drug became detectable at 2 hours rising to 15 uM by 3 hours post injection. Mice receiving

5 pro-drug and CPG2-W14 (Fab$_2$) had pro-drug levels of 2.8 uM at 5 mins post injection and 0.5 uM at 60 mins; active drug was detected at 200 uM at 5 mins falling to 2 uM at 3 hours.

Whether CPG2-W14 (Fab$_2$) was given 24 hours or 48

10 hours before there was at 5 mins after pro-drug administration, a 10-fold higher concentration of active drug in the plasma than there was even after 150 minutes in controls not receiving CPG2-W14(Fab$_2$) showing that conversion of pro-drug to active drug occurred efficiently

15 in vivo in the presence of the antibody enzyme conjugate.

EXAMPLE 4

Distribution of Pro-drug and Active Drug in vivo (Plasma and Tumour

Group 1 mice received pro-drug at 41 uM/kg and were bled,

20 sacrificed and tissues removed at 5, 15, 30, 60, 120, 240 mins later.

Group 2 mice received active drug of equivalent molarity (41uM/kg) otherwise as group 1.

Group 3 mice received 29 units of CPG2-W14 (Fab$_2$)

25 conjugate 48 hours before pro-drug as per group 1.

- 26 -

Plasma:

In group 1 the peak concentration of pro-drug 30 uM fell to 2 uM at 120 mins. Active drug first became detectable at 120 min rising to 40 uM at 240 min.

5   In group 3 active drug concentration was 100 uM at 5 min, 150 uM at 30 min and 60 uM at 240 min.

Tumour:

In group 1 the tumour concentration of pro-drug 10 at 5 min was 8 uM falling to 0.4 uM by 240 min; active drug was just detectable at 0.6 uM at 240 min.

In group 2 active drug concentrations were 35 uM at 30 min and 18 uM at 240 min.

In group 3 active drug concentrations varied 15 around 20 uM throughout the study period.

Although the plasma concentration for group 3 is higher than tumour concentration the tumour values do not take account of drug covalently bound to DNA which would not be released by the extraction procedure used.

EXAMPLE 5

Therapeutic Experiment with CC3 Tumour

Nude mice bearing human choriocarcinoma tumour received saline (Group A) or a preparation of the pro-drug 9 mg (Group B) or 22.5 mg (Group C) at 48, 60 and 72 hours after receiving 25 100 units of CPG2-W14 (Fab$_2$). Tumour volumes were

- 27 -

measured in 3 planes and their volumes calculated (Figure 3). Group B mice showed a delay in the onset of tumour growth whilst the higher dose of drug produced complete regression of the tumour.

EXAMPLE 6

5

p-[(2-Mesylethyl)-(2-chloroethyl)-amino]benzoyl glutamide (Compound IV) is prepared by the reaction scheme illustrated above starting from p-bis(2-hydroxyethyl)amino-benzoyl glutamide ethyl ester,

10 itself prepared by reaction of the p-aminobenzoyl glutamide ethyl ester with ethylene oxide by known methods. The bis-2-hydroxyethyl compound is refluxed in pyridine for 10 minutes at $80^{\circ}C$ using a 3:1 (molar) mesyl chloride/dihydroxy compound to give a reaction product

15 which includes 17-20% by weight of the desired diethyl ester of Compound IV. Mass spectrometry for purified diethyl ester indicates a molecular weight of 506. The diethyl ester protecting groups are then removed by treatment first with aqueous sodium hydroxide and the

20 resulting disodium salt then treated with aqueous hydrochloric acid to give a product including Compound IV. Compound IV is isolated from the reaction product by high pressure liquid chromatography in 25% acetonitrile/water containing 1% acetic acid. Compound IV elutes at 306

minutes. The eluted product is shown by thin layer
chromatography to be pure Compound IV. Compound IV was
converted, for further identification purposes, into its
dimethyl ester and mass spectrometry indicated a molecular
5 weight of 478.

The corresponding bis-(2-mesylethyl) compound
was prepared by a similar method in which the reflux with
mesyl chloride was carried out at $2^{o}C$ for 20 minutes in
pyridine using a 3:1 (mole) mesyl sulphate/dihydroxy
10 compound.

The corresponding bis-(2-trifluoromesylethyl)
compound can be prepared by a similar method in which the
mesyl chloride is replaced by tetrabutylammoniumtrifluoro-
methane sulphonate, $(C_4H_9)_4 \, N(CF_3SO_3)$.

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- 31 -

WE CLAIM:

1.   A two component system designed for use in association with one another comprising

(i)   a first component that is an antibody fragment capable of binding with a tumour associated antigen, the antibody fragment being conjugated to an enzyme capable of converting a cytotoxic pro-drug into an cytotoxic drug.

(ii) a second component that is a cytotoxic pro-drug convertible under the influence of the enzyme to the cytotoxic drug.

2.   A system according to claim 1 wherein the antibody is a monoclonal antibody.

3.   A system according to claim 1 or 2 wherein the antibody is of the IgG class.

4.   A system according to any one of the preceding claims wherein the antibody fragment is a $F(ab')_2$ fragment.

5.   A system according to any one of the preceding claims wherein the enzyme is of non-mammalian origin.

6.   A system according to any one of the preceding claims wherein the enzyme is a carboxypeptidase.

7.   A system according to any one of the preceding claims wherein the pro-drug is an amide obtainable by reaction of a disubstituted amino benzoic acid nitrogen mustard and an amino acid.

8.   A system according to claim 7 wherein the amino acid is glutamic acid.

9.   A system according to claim 7 wherein the amino

- 32 -

acid is a D-amino acid.

10. A system according to any one of claims 7 to 9 wherein the nitrogen mustard is p-bis(2-chloroethyl)amino-benzoic acid.

11. A system according to any one of claims 7 to 9 wherein the nitrogen mustard is p-[(2-chloroethyl)-(2-mesylethyl)amino] benzoic acid or p-bis(2-mesylethyl)amino-benzoic acid or p-bis(2-trifluoromesylethyl)amino-benzoic acid.

12. A system according to any one of the preceding claims for use in a method of controlling the growth of neoplastic cells in a host involving administration to the host of first components and subsequently, of the second component.

13. A method of controlling the growth of neoplastic cells in a host which comprises administering to the host a first component as defined in any one of claims 1 to 6 followed by administration to the host of a second component as defined in any one of claims 7 to 11.

14. A method according to claim 13 wherein each component is administered intravenously...

15. A nitrogen mustard pro-drug of the formula

$$M \!\!-\!\!\left\langle\!\!\bigcirc\!\!\right\rangle\!\!-\!\! CONHR$$

where R is the residue of an α-amino acid RNH_2 and M is a

$$-N \begin{cases} CH_2CH_2Cl \\ CH_2CH_2SO_3CH_3 \end{cases}$$

$$-N \begin{cases} CH_2CH_2SO_3CH_3 \\ CH_2CH_2SO_3CH_3 \end{cases}$$

or $\quad -N \begin{cases} CH_2CH_2SO_3CF_3 \\ CH_2CH_2SO_3CF_3 \end{cases}$ group.
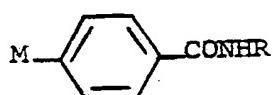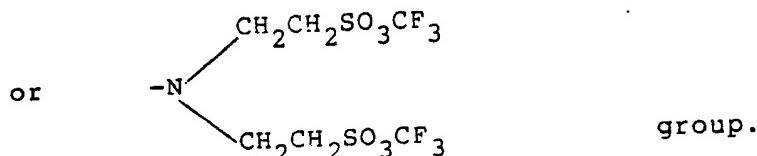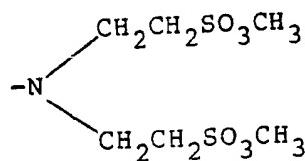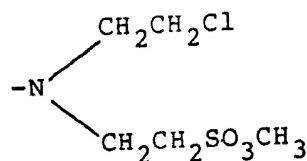
16. A pro-drug according to claim 15 wherein R is a glutamic acid residue.

17. A pro-drug according to claim 15 or 16 where R is the residue of a D-amino acid.

18. A pro-drug according to claim 15 hereinbefore specifically mentioned.

19. A process for preparing a compound as defined in claim 15 which comprises reacting a nitrogen mustard of

– 34 –

formula M⟨benzene ring⟩COOH   or a reactive
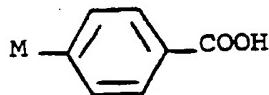
carboxy derivative thereof with a carboxy protected amino

acid $R\,NH_2$ and removing the carboxy protecting group.

20. A process for preparing a compound as defined

inclaim 15 which comprises reacting a compound of the

formula:

$(HOCH_2CH_2)_2N$⟨benzene ring⟩CONHR
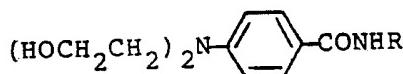
with a reagent capable of replacing the HO group by Cl,

CH_3SO_3 or CF_3SO_3.

21. A pharmaceutical composition comprising a

pro-drug according to any one of claims 15 to 18 together

with a pharmaceutically acceptable carrier or diluent.

22. A composition according to claim 21 suitable

for intravenous administration.

23. A conjugate of carboxypeptidase G2 and the

$F(ab')_2$ fragment of W14A.

Fig.1.

1/3
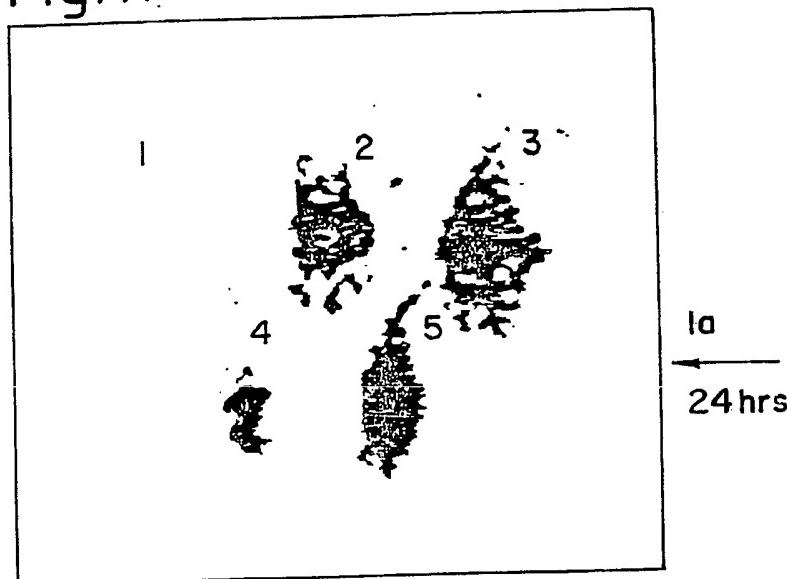
Plates 1a and 1b   Nude rats bearing CC3 choriocarcinoma xenografts on their flanks (arrowed) were injected i.v. or i.p. with SPDP- or MBS-linked W14A:CPG2 conjugates; a fifth tumour-bearing rat control was injected with native ^{131}I-labelled CPG2. 1. Native CPG2, 47µCi, 49µg protein, i.v.

2. W14A:CPG2 (SPDP-linked) 15.3µCi, 63µg protein, i.v.

3. W14A:CPG2 (MBS-linked) 16.5µCi, 77µg protein, i.v.

4. W14A:CPG2 (SPDP-linked) 15.3µCi, 63µg protein, i.p.

5. W14A:CPG2 (MBS-linked) 16.5µCi, 77µg protein, i.p.

SUBSTITUTE SHEET

Fig.2.

DAY 1 — 2a

DAY 3 — 2b
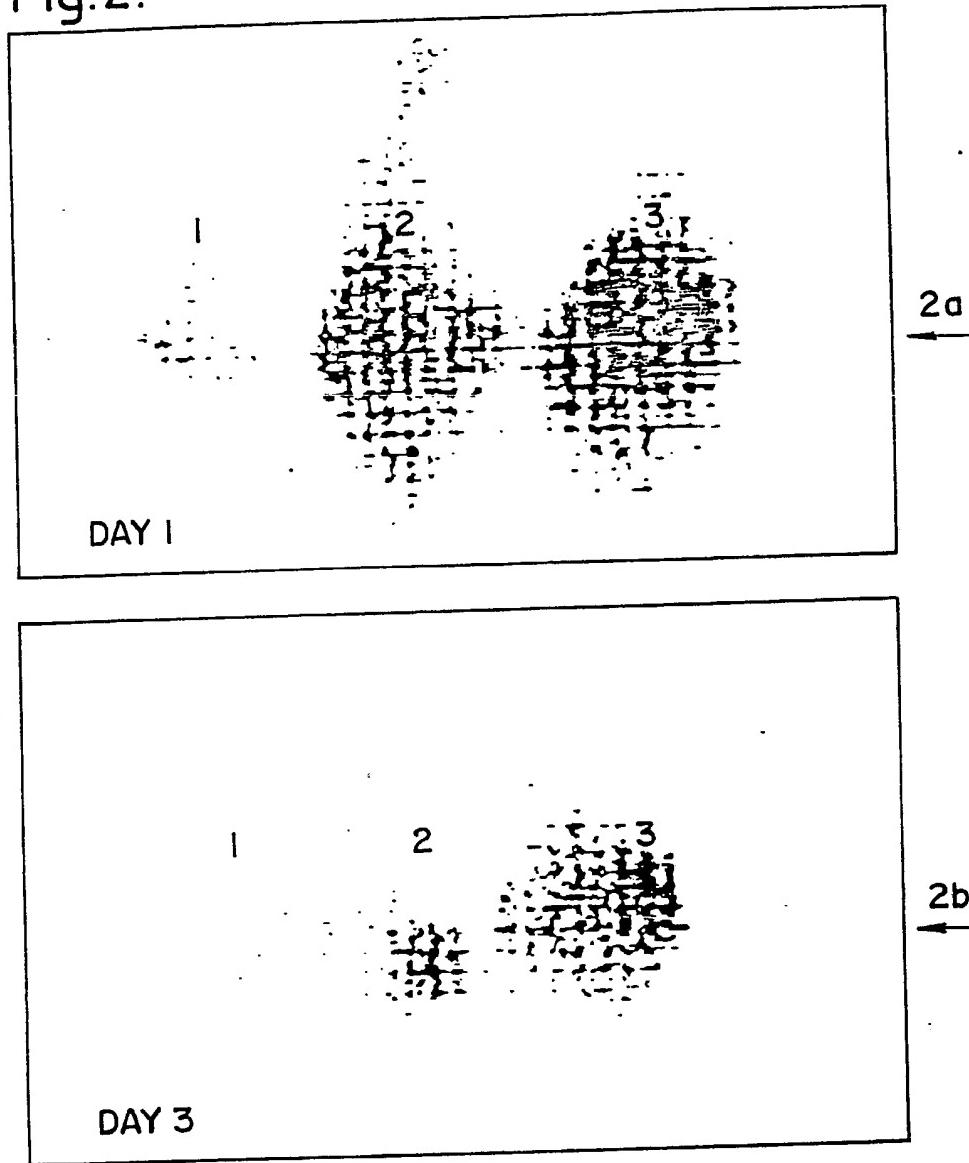
Plates 2a and 2b Nude rats bearing CC3 choriocarcinoma xenografts were injected i.v. with SPDP- or MBS-linked F(ab')$_2$:CPG2 conjugates; a third tumour-bearing rat control was injected with native ^{131}I-labelled CPG2. 1. Native CPG2, 43μCi, 41μg protein, i.v. 2. F(ab')$_2$:CPG2 (SPDP-linked) 17.8μCi, 49μg protein, i.v. 3. F(ab')$_2$:CPG2 (MBS-linked) 16.1μCi, 47μg protein, i.v.

Fig.3. 'Therapeutic' experiment.

CC3 tumour   CPG₂—WI4 (Fab₂)

3 doses of prodrug at 12 hour intervals starting 48 hours post CPG₂—WI4 (Fab₂)
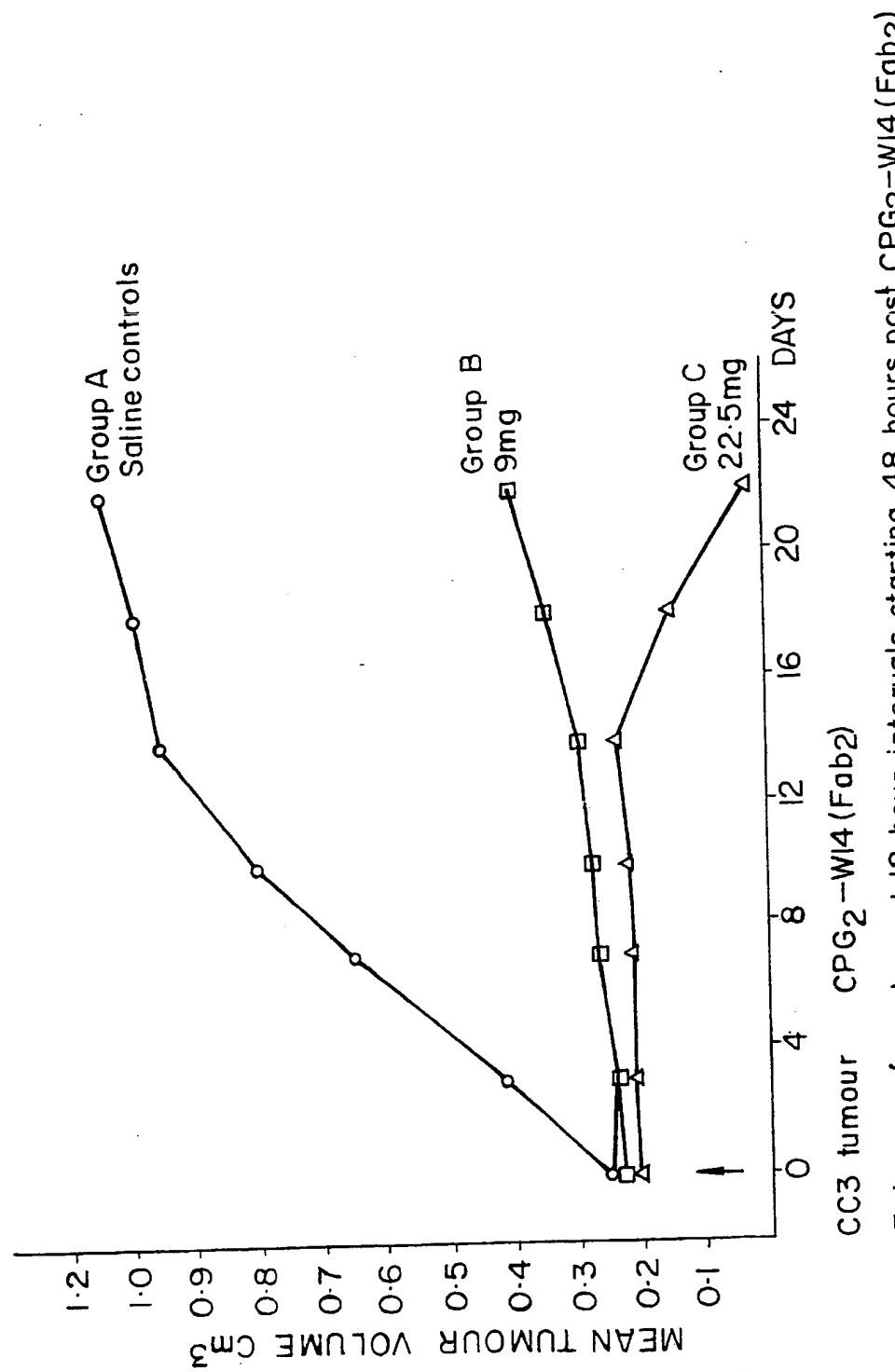
INTERNATIONAL SEARCH REPORT

International Application No **PCT/GB 88/00181**

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC^4: A 61 K 39/395; A 61 K 45/05; //(A 61 K 39/395, 31:165)

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC^4	A 61 K

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched 8

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X	FR, A, 2584294 (P. BERDAL) 9 January 1987 see claims 1,2,15	1,2,5-7
X	Dialog 05464842, 85080842 Medline, A.R. Ahmed et al.: "Cyclophosphamide (Cytoxan). A review on relevant pharmacology and clinical uses", see abstract & J. Am. Acad. Dermatol., Dec 1984, 11(6) p1115-26	1,7

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* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18th August 1988	29. 08. 88

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.

GB 8800181
SA   21051

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The members are as contained in the European Patent Office EDP file on 26/08/88
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
FR-A- 2584294	09-01-87	None	

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82